THE DEVELOPMENTAL HISTORY OF AMAROECIUM CONSTELLA-
TUM. 1. EARLY EMBRYONIC DEVELOPMENT

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INTRODUCTION

The Tunicata have held the interest of biologists since Kowalevsky established their taxonomic position by identifying the larvae with Chordates. In their embryology apparent mosaic development has presented a rich field of investigation for both descriptive and experimental embryology. Conklin's exhaustive study of Styela (1905) established the pattern of development which has been verified for Ascidiella, Ciona, Molgula, Botryllus, Phallusia, and also for Amphioxus. These forms agree in having a moderate supply of yolk that neither obscures nor interferes with the pattern of development. Studies on the early embryology of heavily yolked forms have been restricted to papers on Distaplia (Davidoff, 1899–01) and *Amaroecium proliferum* (Maurice et Schulgin, 1884). Since these investigations are incomplete it was thought that the study of an egg with abundant yolk might be of interest in analyzing the extent to which yolk modifies the processes of mosaic development. *Amaroecium constellatum* was chosen because of the apparent twisting of symmetry in its axial structures, the neural tube lying to the left of the notochord rather than dorsal to it.[1]

MATERIAL AND METHODS

Amaroecium constellatum is a compound Ascidian commonly known along the eastern shore of the United States as "sea pork." The zooids are elongate and clustered together to form thick fleshy colonies. The gonads are located in the long post-abdomen, testes posterior to the ovaries which crowd up against the lower part of the abdomen. Fertilization is internal and the embryos develop within "brood spaces." The embryos are located along the length of the ascidiozooid according to the degree of development, the eggs and early stages being lodged in the post-abdomen and lower abdomen; the later stages of tadpoles, in the thoracic region from which they escape when development is completed. The shape of the eggs varies by crowding from spherical to polyhedral. The average diameter of the fixed egg is 250 micra.

The breeding season extends throughout the summer months. All stages are abundant during July and August. During the latter part of June and the early part of September the embryos are few in number. Eggs and embryos are obtained by squeezing the adult colonies in a finger bowl of sea water. Some of the

earlier stages may be obtained by dissecting the individual members of colonies under a low power microscope. The post-gastrulation stages will continue to develop in tanks in the laboratory but the pre-gastrulation stages are extremely sensitive. They disintegrate shortly after their removal from the adults without completing the divisions then in progress. These embryos must be used as soon as removed and when the material is fresh.

The study of whole embryos is made difficult but not impossible by the character and arrangement of the test cells. Observations, as far as the establishment of the neural plate, were made on whole specimens. They were fixed in Bouin's fluid and preserved in 70 per cent alcohol in which they were mounted on shallow depression slides in vaseline cells to permit rolling them about for examination. The picric acid is retained strongly by the yolk granules whereas the yolk-free cytoplasm, immediately about the nucleus, is colorless. The contrast provides a reliable means for the identification of cells. The position of the spindles can be ascertained and the orientation of the cells known with certainty. Both reflected and transmitted light were used.

Berrill's (1932) technique of hydrolyzing the test with digestive juices was tried but by the time the closely applied test was removable the enzymes had attacked the cells themselves.

The critical stages immediately preceding and following gastrulation were drawn with the aid of a camera lucida, then embedded and sectioned in the manner suggested by Doctor Eleanor Slifer (oral communication) for yolk-laden eggs. All reconstructions were made from serial sections studied in conjunction with whole cleared specimens.

This study deals with the embryo as far as the end of gastrulation. A second paper is concerned with organogenesis in the tadpole.

EARLY DEVELOPMENT

Since there are many maturation spindles in evidence in the unfertilized eggs but no polar bodies formed, it seems reasonable to conclude that Amaroecium agrees with Styela in extruding its first polar body at fertilization. The egg at this time is plentifully supplied with yolk granules. The test cells are embedded in the peripheral cytoplasm in a compact layer and the follicle cells are tightly pressed against the chorion. After fertilization the test cells are clustered outside the egg and inside the chorion in small irregular groups, some remaining embedded in the cortical cytoplasm. The chorion is lifted from the surface leaving a wide perivitelline space. As cleavage proceeds the test cells completely fill up this space as well as any available cleavage furrows and depressions on the surface of the young embryo. On its external surface the test presents the appearance of a pavement epithelium. In section it consists of compact layers of spindle shaped cells with deeply staining nuclei.

The first cleavage is meridional and divides the egg into two blastomeres which represent the right and left halves of the future embryo. The right blastomere is slightly smaller than the left one (Fig. 1, C; 2, B). In some cases the disparity seems greater than in others, due, probably, to variations in shape of the egg resulting from pressure of contiguous eggs. It has been shown in the simple Tunicates and in Amphioxus (Conklin, 1905, 1932) that the cleavage nucleus lies pos-

terior to the center of the egg but in the midline. The disparity in size of the first two blastomeres in Amaroecium indicates that the meeting of male and female pronuclei is effected not only in the posterior region of the egg but also to the right side of the midline (Fig. 1, B; 2, A). In Amaroecium, therefore, there is a double eccentricity of the zygote nucleus, posterior and to the right of the main axis of the egg.

In all the sections of Amaroecium eggs examined the polar bodies lies eccentrically with respect to the main mass of yolk (Fig. 1, A). By assuming that the germinal vesicle always lies to the right of the apex of the elliptical egg and also that the sperm penetrates on the same side it is possible to explain the lateral eccentricity of the zygote nucleus to the right of the median axis; this, in turn, explains the constant inequality in size of the first two blastomeres.

The second division also is meridional and at right angles to the first, thus dividing the egg into two anterior and two posterior blastomeres. The posterior
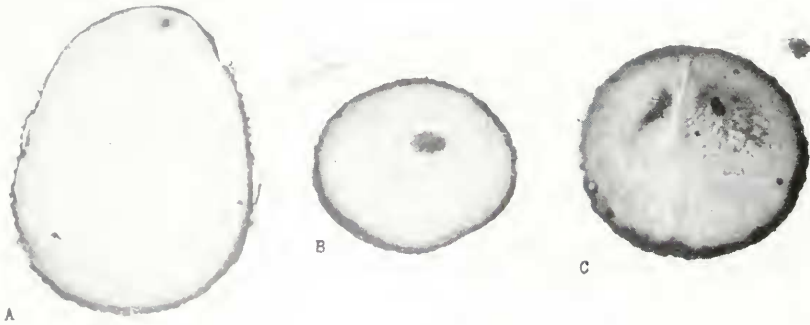

FIGURE 1. Photomicrographs of A. Fertilized egg; first polar body extruded, spindle for second polar body forming. Chorion is broken at the animal pole. Test cells bulge through the chorion. B. Cross section through animal pole of egg after fusion of the pronuclei showing double eccentricity of the nucleus posterior to and lateral to the midplane of the egg. C. Two cell stage; smaller cell is right blastomere. Magnification—250 ×.

blastomeres are smaller than the anterior two. Beginning with the smallest of the four they fall into this order: the right posterior, the right anterior, the left posterior and the left anterior. The nuclei of all lie at the extreme tip of the cells toward the animal pole (Fig. 2, C, D). The third cleavage cleaves the egg in the latitudinal plane into four micromeres at the animal pole and four heavily yolked macromeres at the vegetative pole.

The nuclei and their areas of yolk-free cytoplasm elongate in the direction of the next division and the spindle remnants remain clearly evident after the division is completed. These two features constitute valuable means of identifying the axes of the embryo.

The spindles of the two anterior micromeres are parallel with the antero-posterior axis whereas in the two posterior micromeres they are transverse to this axis and the posterior cells form an arc around the anterior cells (Fig. 3). The elongate cytoplasmic areas of the posterior macromeres in preparation for their next

division converge towards the animal pole. The cytoplasmic areas of the anterior macromeres lie parallel with the lateral borders of the overlying micromeres (Fig. 3).

It will be convenient for purposes of ready reference to designate the cells by the letters used by Conklin in his study of cell lineage in the Tunicates. By underlining the cell designation of the left side he distinguishes them from their corresponding members on the right side. In the eight cell stage the anterior left macromere is A 4.1, the right one is A 4.1. The corresponding micromeres are
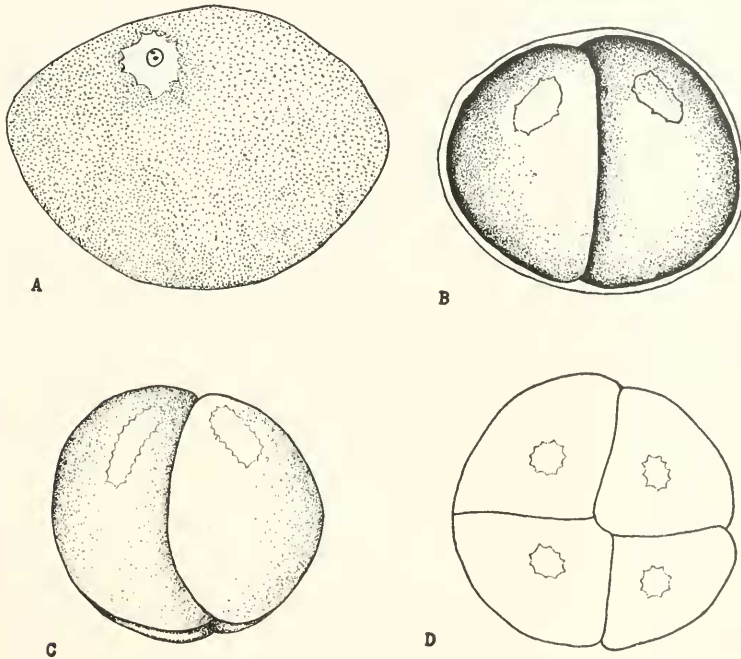

FIGURE 2. A. Fertilized egg before first division, B. Two cell stage; posterior view, C. Four cell stage; anterior view, D. Polar view of four cell stage. Vegetative pole position of nuclei showing. Except where noted all magnifications are 266 ×.

a 4.2 and a 4.2. The posterior left and right macromeres are B 4.1 and B 4.1 respectively. Their corresponding micromeres are b 4.2 and b 4.2 (Fig. 3).

The fourth division is significant in that it accomplishes the distribution of cytoplasmic substances to areas similar to those recognizable in forms with less yolk in their eggs. All the micromeres divide at approximately the same time. Both pairs of macromeres divide unequally into a smaller pair of cells toward the animal pole and a large pair of yolk-charged macromeres occupying the entire vegetative hemisphere. The median derivatives of the anterior pair constitute the chorda-neural crescent, the posterior derivatives are combined mesoderm and ectoderm. All sixteen cells can be seen in polar view (Fig. 4).

Since the cleavages are not synchronous beyond the sixteen cell stage it may be

well at this point to summarize the cell lineage in the egg of Amaroecium. The micromeres, designated by small letters in the figures, produce the ectodermal cells that eventually grow over all the other cells of the embryo. The "A" macromeres give rise to the chorda-neural crescent, A 5.2 and A 5.2, and half of the endodermal quadrant, A 5.1 and A 5.1. The "B" macromeres give rise to the mesodermal crescent, B 5.2 and B 5.2, and the other half of the endodermal quadrant, B 5.1 and B 5.1 (Fig. 4).

The pattern corresponds exactly to the pattern of Ascidian mosaic development in Styela. The generous provision of yolk in the egg of Amaroecium prevents the appearance of a blastocoele cavity and disposes the presumptive mesodermal cells to a position farther towards the animal pole than this crescent of cells occupies in the egg of Styela where the presumptive endoderm cells are much smaller in size. The hereditary pattern of development is not disturbed by the accumulation of yolk. The mechanics of the process are altered but the same relative positions of the presumptive embryonic areas are assumed. The embryonic areas consist of an endo-
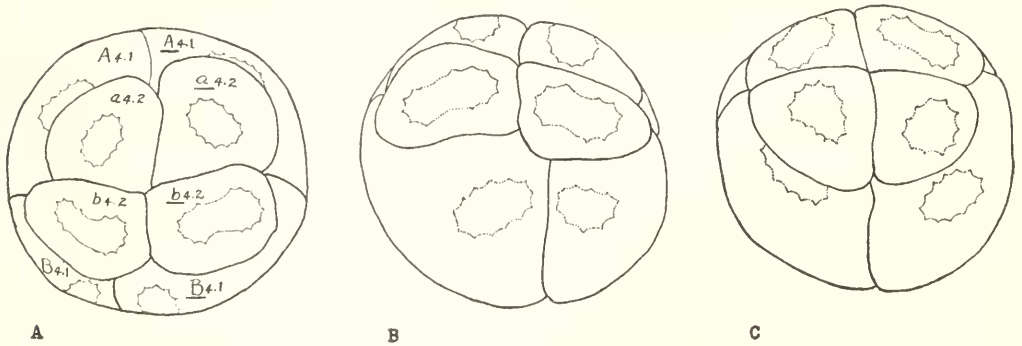

FIGURE 3. Eight cell stage; direction of spindles indicated by stippled areas, A. Animal pole, B. Posterior view, C. Anterior view.

dermal quadrant of large macromeres on the dorsal side, i.e., toward the vegetative pole, with a chorda-neural crescent of two cells lying anterior to them and a mesodermal crescent of two cells lying posterior to them. The ectodermal micromeres occupy the entire animal pole or future ventral side of the embryo. All the cells on the right side are smaller than their sister cells on the left side. Otherwise the cells are disposed symmetrically with respect to the median plane of the embryo.

Before the fifth cleavage the micromeres shift in position; the "a" cells spreading transversely, b 5.4 adjacent to them and b 5.3 overlapping the mesodermal cells (Fig. 5, A). The embryo passes through a twenty-two cell stage in its fifth cleavage. The macromeres and their derivatives divide first, those on the right side preceding those on the left. The mesodermal cells divide meridionally, increasing the number of cells in that arc to four. The posterior macromeres also divide meridionally but unequally, producing two smaller lateral mesodermal cells towards the animal pole and two median elongate macromeres (Fig. 5, C, E). Each of the anterior pair of endodermal macromeres divides into two unequal cells, a smaller one on each side of the chorda-neural crescent and a larger median one (Fig. 5, D).

The four large macromeres meet in a median furrow at the vegetative pole. The chorda-neural cells divide later into a transverse row of four cells.

In the twenty-two cell stage there are three mesodermal cells on the right side, B 6.2, B 6.3, B 6.4 and their corresponding cells on the left (Fig. 5, 6). When they divide at the sixth cleavage the dorsal derivative of B 6.2 which is B 7.4 and of B 6.4 which is B 7.8 are presumptive muscle cells. The ventral members and both derivatives of B 6.3 are mesenchyme. Reference to Figure 5 will show that B 6.3 lies at the mid-region of the posterior lip of the blastopore. At this division the
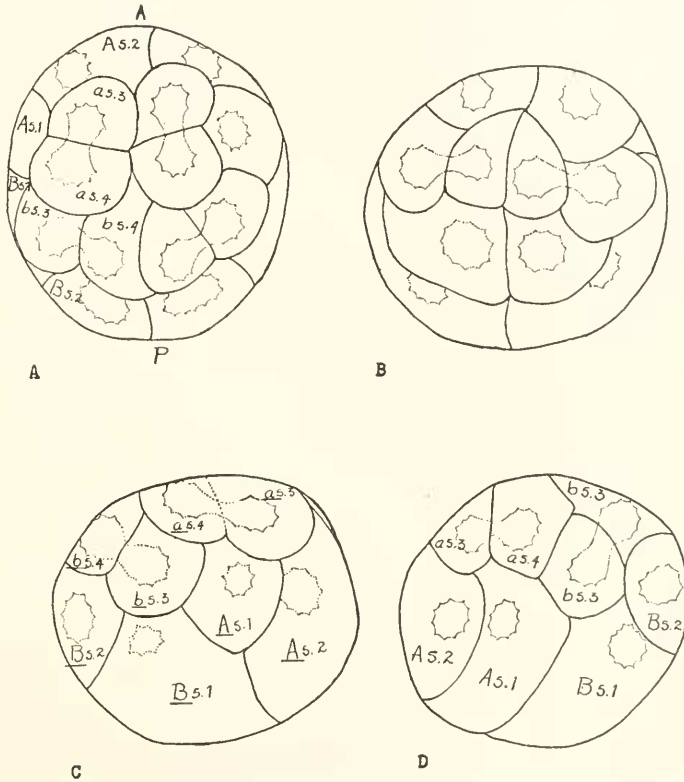

FIGURE 4. Sixteen cell stage, A. Animal pole, cells on right side designated, B. Posterior view, C. Left side, D. Right side.

chorda-neural cells divide into two transverse rows of four neural cells and four chordal cells. The chordal cells lie towards the vegetative pole, the neural cells towards the animal pole and in contact with the ectodermal micromeres (Fig. 7).

No attempt is made beyond this point to follow the lineage of the cells. Since they correspond through the first six cleavages with the cells of Ascidians whose cell lineage can be followed through gastrulation, it may be assumed that their agreement continues through subsequent stages with differences dependent on the mechanics of gastrulation in Amaroecium.

It may be helpful before presenting the process of gastrulation to clarify the terms used in describing it. The blastopore is the margin of cells surrounding the macromeres. Its anterior border consists, at first, of chorda-neural cells and later of neural cells only. It is consistently called the anterior lip (the "dorsal lip" of embryologists dealing with Amphibian forms). Its posterior border ("ventral lip") is the crescent of presumptive mesodermal cells and is called the posterior lip. Lateral regions are referred to as right and left lips respectively.

In Styela, which provides the pattern to be used as a basis for comparison, movements of cells in gastrulation are not modified by accumulation of yolk. The
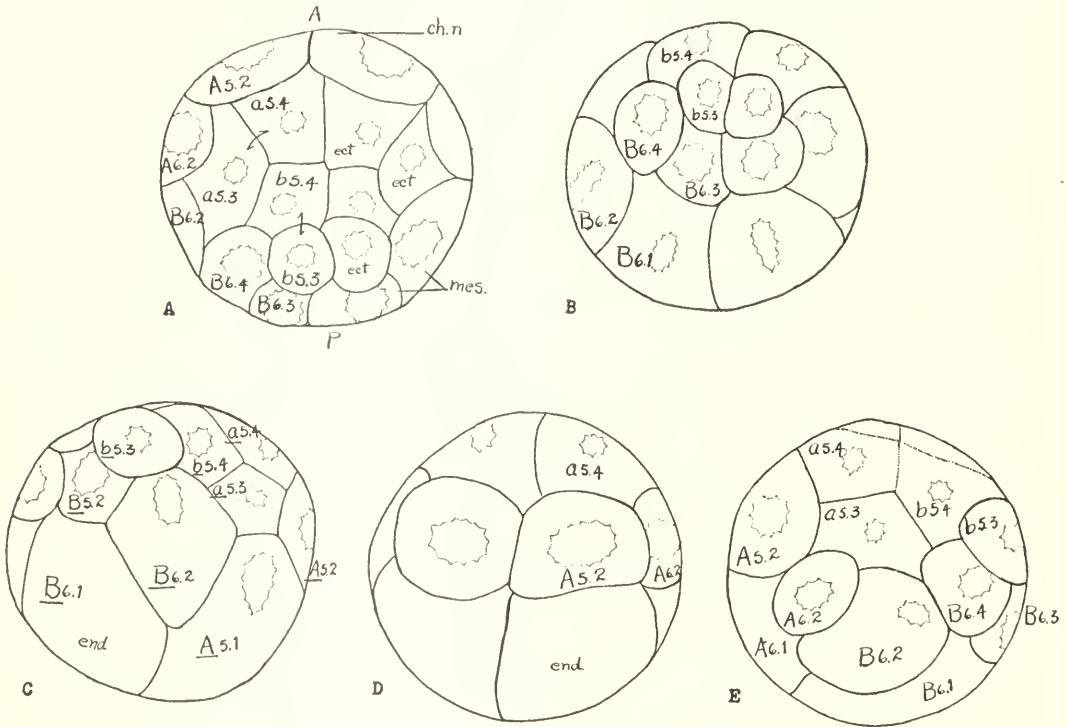

FIGURE 5. Twenty-two cell stage stage.
A. Animal pole; *ch. n.*—chorda-neural crescent; *mes.*—mesodermal crescent; *ect.*—ectodermal micromeres, B. Posterior view, C. Left side, D. Anterior view, E. Right side.

first cells of the mesodermal crescent to be inturned at the lateral lips of the blastopore are mesenchyme cells which come to lie ventrally in the trunk region of the embryo. The cells that converge medially to form the lateral lips after the mesenchyme invaginates are presumptive muscle cells of the tail. The final blastopore is T shaped, neural cells forming the anterior lip, muscle cells forming the lateral margins of the posterior lip. The posterior lip forms the limb of the T where the lateral lips converge toward the mid-plane in a groove. At the posteriormost point in the groove the caudal mesenchyme cells are lodged. Chordal and endodermal cells invaginate, the latter forming a typical archenteron. The blasto-
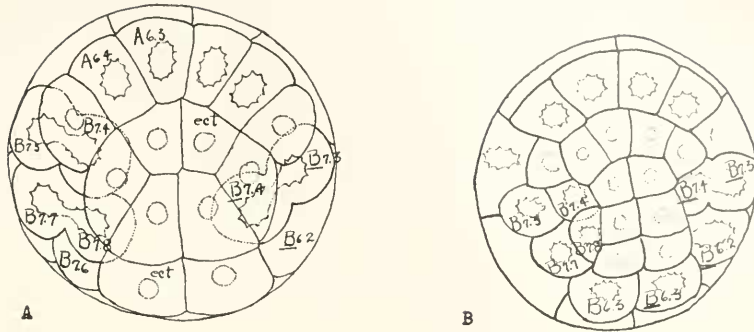
FIGURE 6. A. Thirty-two to sixty-four cell stage; division on the right side in advance of the left side. B. Same stage, mesodermal cells having completed division on the right side but lagging on the left.

pore closes by posterior growth of the anterior lip and growth toward the mid-line of the lateral lips.

The egg of Amaroecium departs from this pattern of gastrulation in several respects. The margins of its blastopore are established at the sixth cleavage. The anterior lip consists of four chordal cells, the posterior lip of mesodermal cells. Enclosed by the blastoporal lips are the large vegetative macromeres (Fig.
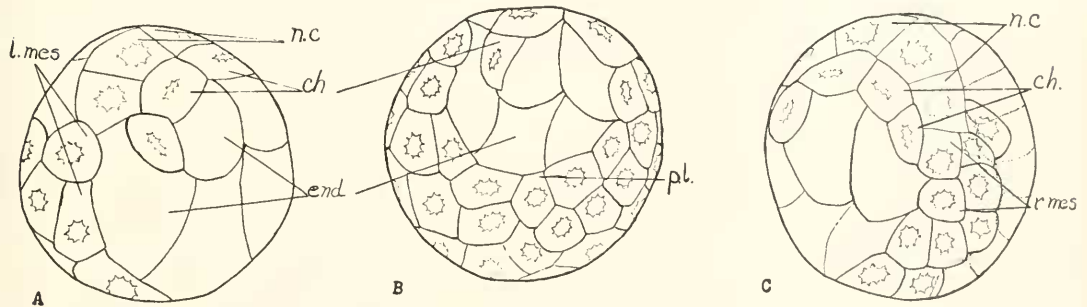

FIGURE 7. Gastrulation.

A. Viewed from the left lateral blastoporal lip, B. The embryo tilted toward the animal pole to show the complete lip of the blastopore. The angle distorts the size of the endodermal area, C. Viewed from the right blastoporal lip; *ch.*—chordal cells; *end.*—endodermal macromeres; *l.mes.*—mesoderm of left lateral lip of blastopore; *n.c.*—neural cells; *r.mes.*—mesoderm of right lip; *p.l.*—posterior lip of blastopore.

7). In subsequent cleavages they divide into cells of unequal size. The cells at the animal pole are fairly uniform (Fig. 5, 6). All the animal micromeres may be called the epiblast. They differentiate into ectoderm which spreads over the embryo by the process of epiboly.

The mass of inert yolk in the macromeres prevents an invagination of the potential endodermal cells. These cells, therefore, do not participate in the early movements of gastrulation. Gastrulation commences with activity of the cells

in the mesodermal crescent. They divide and move over the surface of the macro-
meres in the direction of the vegetative pole. The cells on the right side of the
crescent precede those of the left side in dividing. Smaller size of the macromeres
on the right side and accelerated rate of division of these mesodermal cells effect
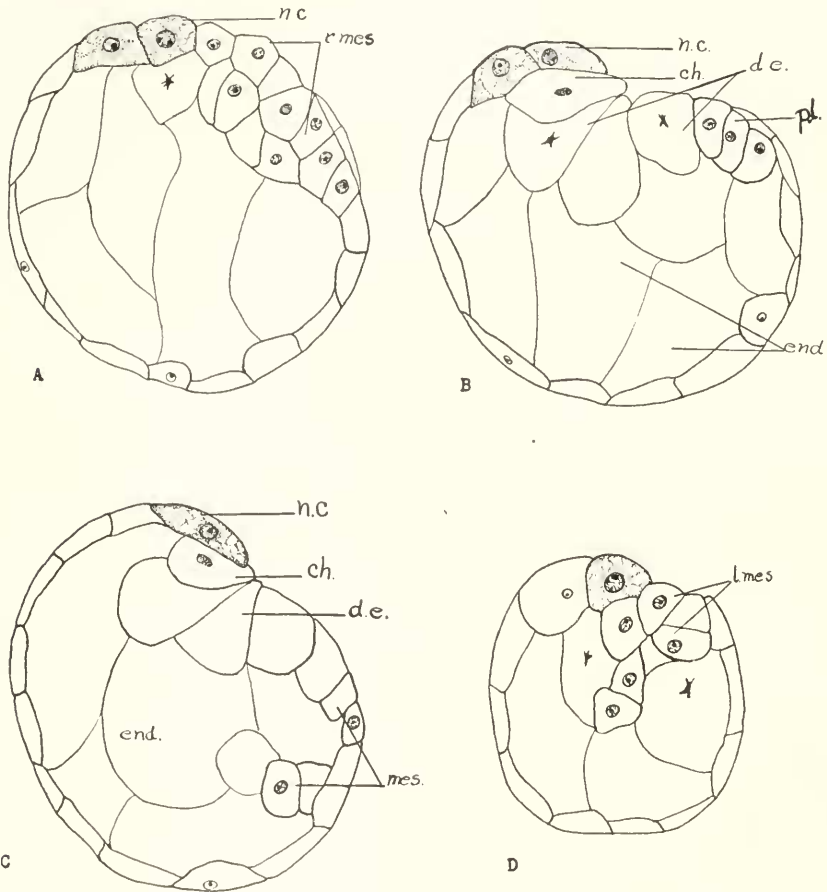

FIGURE 8.   Sections through a gastrula during the period of pseudogastrulation.

A. Through the right lip, B. Through the middle of the blastoporal lip, C. Through the re-
gion immediately to the left of the preceding, showing the mesodermal cells lower on the left
side, D. Through the left lip; depression present where mesoderm meets neural cells; ch.—
chorda; d.e.—definitive endoderm or endoderm of the pharyngeal roof; end.—yolk-laden endo-
derm of floor of pharynx; mes.—mesoderm.

a change in shape of the blastopore from circular to an irregular oval. More
rapid overgrowth of the right lateral region of the posterior margin results in a
narrowing of the blastoporal rim on that side in the antero-posterior direction
(Fig. 7). The cells converge medially to form lateral lips as they do in Styela
but they converge more rapidly from right to left and thus distort the shape of the

blastopore. The right lateral margin defines more of a horizontal curve than the left (Fig. 9, A, B). The cells of both chordal and neural crescents, the anterior lip, increase in number to eight.

As the blastopore becomes smaller changes occur in the endodermal area. The macromeres divide into a number of polyhedral cells, those at the surface of the region enclosed by the blastopore being smaller than those constituting the internal yolk mass (Fig. 8, B, C). This layer of cells may be called the endodermal plate. Corresponding in shape with the blastopore it is a small oval region tapering to a narrow point on the anterior right side where the blastoporal lips are approaching each other more rapidly than they are on the left side. The cells of the endodermal plate change in shape from polyhedral to pyramidal, their apices tapering into a cleft-like depression on the surface formed simultaneously with their
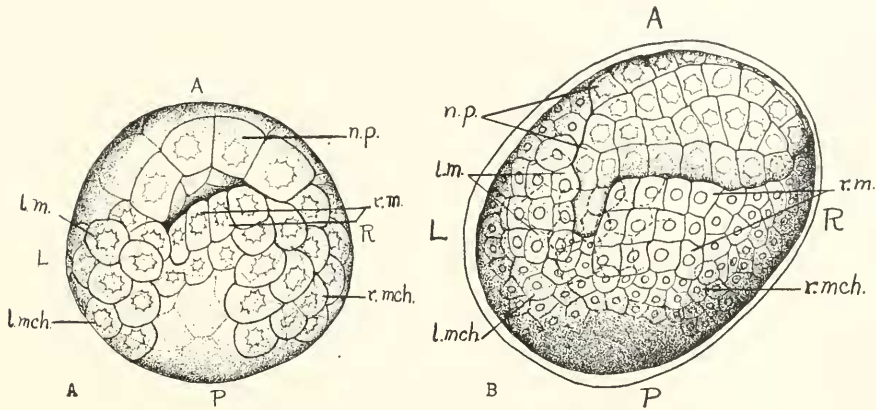

FIGURE 9. Late gastrulation.

A. Dorsal view. Blastopore closed on right side when depression appears in endoderm. Mesoderm growing from right to left in lip of blastopore. B. Posterior view of embryo at the end of the gastrulation period, tilted slightly to show complete left side. Cells with interrupted outlines represent ectoderm, shown only where blastopore has closed; *l.m.*—left muscle cells; *l.mch.*—left mesenchyme; *n.p.*—neural plate; *r.m.*—right muscle cells; *r.mch.*—right mesenchyme.

change in shape. Their broad bases rest on the larger endodermal cells in the interior of the embryo (Fig. 8, B, C). Such an invagination may be called a "pseudo-invagination" since it closes again without the formation of an archenteron.

The chordal cells adjacent to the endodermal plate invaginate with the endodermal cells. They are involuted at the anterior lip of the blastopore and come to lie immediately underneath the neural plate and dorsal to the endoderm (Fig. 8, A, B, C). With involution of the chordal cells the blastopore closes on the right side. The mesodermal cells of the posterior margin on this side now occupy their internal embryonic position (Fig. 8 A). The ectodermal cells have overgrown them and the ectodermal cells meet the neural cells at the right side of the neural plate. The remaining mesoderm curves around the region of "pseudo-invagination" to the left end of the neural plate or anterior lip where the mesoderm has

been proliferating more slowly. The lateral margins of the posterior blastoporal lip are potential muscle cells of the tail; the central region is caudal mesenchyme.

The relationship of the blastopore regions are the same as they are in gastrulation stages of Styela where the blastopore is finally T shaped. Convergence of the lateral margins toward the median axial plane is asymmetrical and the lateral margins fuse to the left of the mid-line. The blastopore of Amaroecium may be described as an irregular T the right horizontal bar of which is longer than the left (Fig. 9, A, B).

Closing of the blastopore on the right side produces a slight horizontal curve in the neural plate. When the depression in the endodermal plate closes, the cells of the neural plate extend posteriorly. The neural cells slope gradually, the left side lying more posteriorly than the right side (Fig. 10).
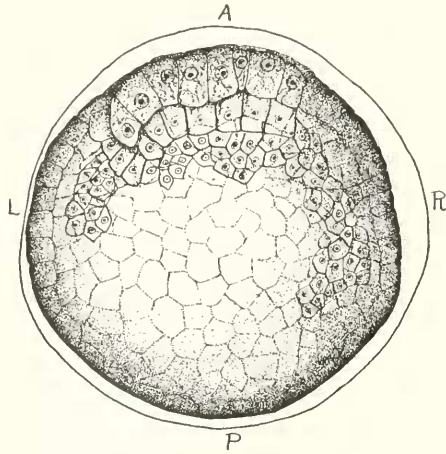

FIGURE 10. Embryo at end of gastrulation. Posterior row of neural cells showing drawn toward mesoderm on left side. Ectoderm in stippled lines; mesenchyme small cells in masses on right and left sides; presumptive muscles stretched across lip and to left of lip.

The activities of all marginal cells in closing the blastopore correspond to the same activities in other Tunicates but the difference in rhythm of division between the cells on the right and the cells on the left deflects the posterior margin of the blastopore to the left (Fig. 8, D). This asymmetrical growth shifts the right muscle cells to the dorsal side of the notochord, the left muscle cells to the ventral side. Convergence and fusion of the cells of the blastoporal margins to the left of the mid-line results in the posterior extension of the neural plate laterally instead of mid-dorsally. The neural tube lies, therefore, to the left of the notochord instead of dorsal to it. Amaroecium is the only Tunicate known in which there is a twisting of the neural tube from a position dorsal to the notochord.

When gastrulation is completed the embryo is approximately spherical with a rudiment of a tail. It lacks an archenteron and the posterior region of the neural plate or potential neural tube is asymmetrical, curving through an angle 90° to the left. Except for these two differences it resembles superficially at this early stage the tadpole of other Ascidians.

Discussion

All the Tunicates whose embryology is known conform to a pattern of mosaic development. *Amaroecium constellatum* despite the fact that its egg is heavily yolk-laden follows the same pattern with modifications contingent upon the mechanical interference of yolk. As Conklin has pointed out, "Cleavage is less constant and fundamental than the type of localization and the two may be relatively independent."

The ooplasmic substances are distributed to cells that assume the same relationships found in forms not filled with yolk. The main mass of inert yolk remains at the vegetative pole and spreads the early blastoporal lips into a circle wider than that of Styela or Amphioxus although the cells are disposed in the same pattern. Ectodermal micromeres occupy the animal pole, yolk occupies the vegetative pole, and between these two areas lie an anterior crescent of mesodermal cells constituting the lip of the blastopore.

Three movements concur in carrying the cells to their final positions where they differentiate into the fundamental structures of the adult body, involution or invagination, epiboly, and convergence or the movement of axial structures into their positions in the median plane. Invagination of the endoderm is impossible but what might be considered an abortive attempt at invagination is made in the depression of the definitive endoderm. It may be called "pseudo-invagination." The depression is accomplished by a change in shape of the endodermal cells whereby they are depressed below the surface. It is not an invagination of cells into a segmentation cavity. Neither does it effect the formation of an open archenteron.

As the mesoderm proliferates over the surface of the endoderm the epiblast cells grow over them. By the process of epiboly, therefore, both mesoderm and endoderm are established in their typical relationships. Since the mesoderm continues to spread between ectoderm and endoderm its movement may be regarded as invagination.

Convergence is the process most violently disturbed by the modified pattern of mosaic development. Its disturbance is due primarily to the bilateral inequality in the size of the first two blastomeres which is responsible for difference in rhythm of division between the right and left halves of the embryo. Cell movements of gastrulation, being greater on the right side than on the left, the blastopore becomes asymmetrical in shape. Cells are thus prevented from converging towards the mid-plane. The lateral margins of the posterior border of the blastopore converge and close on the left side and the posterior extension of neural cells curves through a gradual angle to 90° from the mid-dorsal plane.

Dalcq (1938) concludes that the notochord does not induce neural tube formation in the Protochordates as it does in Amphibia (Spemann, 1928). The notochord in Amaroecium is axial and cannot be responsible for the normal asymmetry of the visceral ganglion and neural tube. There is no experimental evidence in the Ascidians to support the dependence of differentiation of the neural tube on presumptive mesodermal tissue. The fact that the asymmetry of the posterior parts of the nervous system in Amaroecium follows the asymmetry of the mesoderm at the blastoporal lip may indicate some degree of dependence between the mesoderm and the differentiation of the nervous system of the trunk in Protochordates.

SUMMARY

1. The egg of Amaroecium contains more yolk than that of any of the other Ascidians whose embryology has been studied.

2. In the two cell stage the right blastomere is always smaller than the left, establishing an inequality in size that persists through subsequent divisions.

3. In the fifth division the cytoplasmic substances are distributed in this fashion: ten ectodermal cells at the animal pole, four endodermal macromeres at the vegetative pole, a crescent of two chorda-neural cells between them on the anterior side, a crescent of six mesodermal cells on the posterior side, two endodermal-mesodermal cells on each side.

4. Decreased size of cells and increased activity in the mesodermal cells on the right side produce asymmetry in the blastoporal lip.

5. Gastrulation is accomplished by the combined processes of overgrowth and invagination.

6. The blastopore closes from right to left, producing a curve in the neural plate through an angle of 90° in the region of the potential neural tube.

7. When the blastopore closes the potential muscle cells lie above and below the notochord, interrupted on the left by the neural tube, on the right by the endodermal rod.

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